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Nicotine metabolism, human drug metabolism polymorphisms, and smoking behaviour

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Abstract

Large interindividual differences occur in human nicotine disposition, and it has been proposed that genetic polymorphisms in nicotine metabolism may be a major determinant of an individual's smoking behaviour. Hepatic cytochrome P4502A6 (CYP2A6) catalyses the major route of nicotine metabolism: C-oxidation to cotinine, followed by hydroxylation to *trans*-3′-hydroxycotinine. Nicotine and cotinine both undergo *N*-oxidation and pyridine *N*-glucuronidation. Nicotine *N*-1-oxide formation is catalysed by hepatic flavin-containing monooxygenase form 3 (FMO3), but the enzyme(s) required for cotinine *N*-1′-oxide formation has not been identified. *trans*-3′-Hydroxycotinine is conjugated by *O*-glucuronidation. The uridine diphosphate-glucuronosyltransferase (UGT) enzyme(s) required for *N*- and *O*-glucuronidation have not been identified. CYP2A6 is highly polymorphic resulting in functional differences in nicotine C-oxidation both in vitro and in vivo; however, population studies fail to consistently and conclusively demonstrate any associations between variant *CYP2A6* alleles encoding for either reduced or enhanced enzyme activity with self-reported smoking behaviour. The functional consequences of FMO3 and UGT polymorphisms on nicotine disposition have not been investigated, but are unlikely to significantly affect smoking behaviour. Therefore, current evidence does not support the hypothesis that genetic polymorphisms associated with nicotine metabolism are a major determinant of an individual's smoking behaviour and exposure to tobacco smoke.
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Keywords: Nicotine; Metabolism; Genetic polymorphism; Smoking behaviour

1. Introduction

The hypothesis that nicotine may have a specific role in tobacco dependence and regulating smoking behaviour (Russell and Feyerabend, 1978) has resulted in considerable interest in using nicotine and its metabolites as biomarkers of changes in smoking behaviour (reviewed by Scherer, 1999). The relatively short half-life for nicotine ($t_{1/2} \sim 2.6$ h) precludes its use as an accurate marker of the actual amount of nicotine absorbed from cigarette smoke (Benowitz and Jacob, 1993). Since cotinine is the principal proximate metabolite of nicotine, and has a half-life of about 15–17 h, it has been assumed to be a better biochemical measure of nicotine uptake (Benowitz and Jacob, 1993).

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Typically, 70–80% of nicotine is converted to cotinine prior to metabolism to other metabolites (Benowitz et al., 1994). However, large interindividual differences in the extent of nicotine metabolism to cotinine and in the clearance of cotinine limit the accuracy of blood cotinine levels as an indicator of nicotine exposure and, consequently, smoking behaviour (Adlkofer et al., 1989; Benowitz et al., 1994). Since blood and salivary cotinine concentrations are highly correlated (Jarvis et al., 1988; Curvall et al., 1990; Wewers et al., 2000; Zevin et al., 2000), the same limitations also apply to using salivary cotinine measurements to assess smoking behaviour. Although urinary cotinine levels distinguish between smokers and nonsmokers, they are not a reliable quantitative measure of the extent of cigarette smoke exposure (Yang et al., 2001). As urinary cotinine levels account for less than 15% of the total dose of systemic nicotine, even a small perturbation in the metabolism of nicotine to cotinine could have a large effect on predicting total systemic nicotine exposure using cotinine as a biomarker (Seaton and Vesell, 1993).

Several cytochrome P450 (CYP) enzymes have been identified which mediate in vitro mammalian metabolism of nicotine to cotinine (Flammang et al., 1992; McCracken et al., 1992; Nakajima et al., 1996a). Since most of the identified CYP enzymes are polymorphic, it has been proposed that genetic polymorphisms may influence nicotine metabolism and contribute to differences in an individual's smoking behaviour (Benowitz and Jacob, 1997; Sellers, 1998; Idle, 1999). This hypothesis is based on the premise that a smoker engages in smoking behaviour in such a way as to maintain plasma nicotine levels in a constant range (Benowitz et al., 1989), and the disposition of nicotine will affect smoking behaviour as well as exposure to other tobacco smoke constituents. Thus, genetic polymorphisms associated with reduced enzyme activity should result in compromised depletion of plasma nicotine concentrations and, as a consequence, reduced smoking behaviour. This review summarises environmental and genetic factors affecting drug metabolism enzymes involved in nicotine metabolism and their potential influence on smoking behaviour.

2. Metabolism of nicotine

The major pathways of nicotine metabolism are summarised in Fig. 1. Nicotine is metabolised primarily by C-oxidation to cotinine, and to a lesser extent by N-oxidation to nicotine *N*-1′-oxide, *N*-demethylation, and *N*-glucuronidation. Cotinine is further metabolised by hydroxylation to *trans*-3′-hydroxycotinine and 5′-hydroxycotinine, N-oxidation to cotinine *N*-1-oxide, and *N*-glucuronidation. *trans*-3′-Hydroxycotinine is further metabolised by *O*-glucuronidation. Several additional minor metabolites have also been identified which probably account for less than 10% of total nicotine metabolism (Gorrod and Scherer, [illegible]). Since the determination of urinary nicotine, cotinine, *trans*-3′-hydroxycotinine and their corresponding glucuronic acid conjugates account for approximately [illegible] total nicotine uptake (Table 1), their simultaneous determination provides a better estimate of total nicotine uptake and exposure than the measurement of a single metabolite (Boswell et al., 2000).

The disposition kinetics of nicotine, cotinine, and *trans*-3′-hydroxycotinine are well characterised in healthy smoking volunteers (Scherer et al., 1988; Kyerematen et al., 1990; Benowitz and Jacob, 1994, 2000; Benowitz et al., 1999, 2002; Zevin et al., 2000). The metabolic clearance of nicotine is slower in smokers than in nonsmokers (Kyerematen et al., 1990; Benowitz and Jacob, 1993, 2000). Although this effect has been attributed to inhibition of nicotine metabolism by some component of tobacco smoke, no direct evidence exists to support this hypothesis (Benowitz and Jacob, 2000). Total and nonrenal clearance of nicotine are, on average, higher in Caucasians than in African–Americans (Pérez-Stable et al., 1998) and Chinese–Americans (Benowitz et al., 2002). In healthy subjects, neither cigarette consumption nor type of cigarette smoked can explain ethnic differences in nicotine metabolism. Total and nonrenal clearance of nicotine are decreased in subjects with either alcohol-induced liver cirrhosis (Langmann et al., 2000) or kidney failure (Molander et al., 2000).

Intravenous infusion experiments with abstinent adult smokers show mean plasma elimination half-

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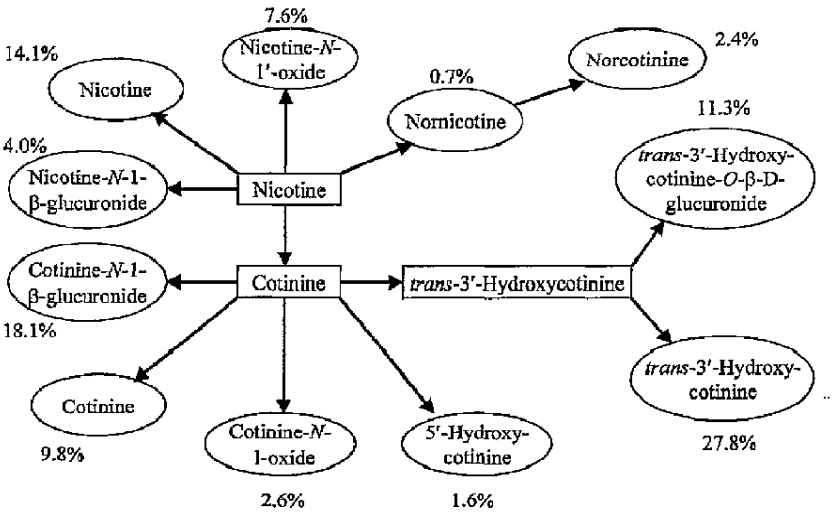

Fig. 1. Quantitative scheme of nicotine metabolism, based on average excretion of metabolites as percentage of total urinary nicotine (Byrd et al., 1995b). Compounds in square boxes detected in blood, circled compounds indicate major metabolites excreted in urine.

lives of 2.3 h (range, 1.6–2.8 h) for nicotine (Benowitz and Jacob, 1994), 17.5 h (range, 8.1–29.3 h) for cotinine (Benowitz and Jacob, 1994), and 6.6 h (range, 4.6–8.3 h) for *trans*-3′-hydroxycotinine (Benowitz and Jacob, 2001). No statistically significant differences are apparent in the disposition of nicotine in elderly subjects compared with younger adults (Molander et al., 2001), and in men and women (Benowitz and Jacob, 1994; Pérez-Stable et al., 1998). Different phases of the menstrual cycle have no effect on nicotine and cotinine pharmacokinetics in premenapausal women (Gourlay et al., 2002). However, clearance of nicotine and cotinine in pregnant women are significantly higher (60 and 140%, respectively), and the mean plasma half-life of cotinine is shorter

Table 1
Reported mean urinary excretion as a molar percentage (% ±S.D.) of total recovered nicotine and metabolites in smokers urine

Study (number of subjects)	Study 1 (11)	Study 2 (12)	Study 3 (91)	Study 4 (12)	Study 5 (5)
Cotinine	13.2±3.9	13.3±3.1	9.2±2.6	14.8±5.9	15.2
Nicotine	10.4±3.7	10.4±4.4	9.4±5.7	7.9±4.6	9.5
trans-3′-Hydroxycotinine	35.2±7.4	39.1±12.5	36.1±10.6	42.4±12.8	34.1
Cotinine-*N*-glucuronide	17.5±6.3	15.8±7.8	14.0±5.4	12.1±6.0	20.1
Nicotine-*N*-glucuronide	2.8+2.2	4.6±2.9	4.5±2.5	2.6±2.1	3.7
trans-3′-Hydroxycotinine-*O*-glucuronide	8.5±3.8	7.8±5.9	22.8±10.0	10.3±7.6	7.4
Nicotine *N*-1′-oxide	6.8±2.9	3.7±0.9	3.0±2.1	N.D.	6.7
Cotinine *N*-1-oxide	3.9±1.9	4.5±1.5	0.9±0.9	N.D.	2.2
Nornicotine	–	0.6±0.2	–	–	–
Norcotinine	1.5±0.5	N.D.[a]	N.D.	N.D.	1.3
Others	–	–	–	10.1[b]	–
Sum	99.8	99.8	99.9	100.2	100.2

85-95%

Study 1 (Byrd et al., 1992), Study 2 (Benowitz et al., 1994), Study 3 (Andersson et al., 1997), Study 4 (Hecht et al., 1999), Study 5 (Meger et al., 2002).

[a] N.D., not determined.

[b] Summation of 4-hydroxy-4-(3-pyridyl)butanoic acid and 4-oxo-4-(3-pyridyl)butanoic acid.

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(8.8 vs. 16.6 h, $P < 0.01$) than postpartum (Dempsey et al., 2002). The mean blood half-lives of nicotine and cotinine in newborns are 11.2 and 16.3 h, respectively, (Dempsey et al., 2000). The mean elimination half-lives in newborns are 9.0, 22.8 and 18.8 h for nicotine, cotinine and *trans*-3′-hydroxycotinine, and 13.0, 19.8 and 19.4 h for conjugated nicotine, conjugated cotinine and conjugated *trans*-3′-hydroxycotinine, respectively, (Dempsey et al., 2000). The average urinary elimination half-life for conjugated *trans*-3′-hydroxycotinine in adult smokers is 7.2 h (range, 4.6–9.4 h) (Benowitz and Jacob, 2001).

2.1. Metabolism by C-oxidation

C-Oxidation of nicotine to cotinine occurs via a two-step mechanism: CYP-mediated oxidation of nicotine to the nicotine-Δ-1′(5′)-iminium ion (Murphy, 1973), followed by conversion to cotinine by cytosolic aldehyde oxidase (Gorrod and Hibberd, 1982). The initial transformation of nicotine to the nicotine-Δ-1′(5′)-iminium ion appears to be rate-limiting (Brandänge and Lindblom, 1979). Large 30-fold differences are evident in the Michaelis–Menten kinetics for in vitro metabolism of nicotine to cotinine by human liver microsomes (Messina et al., 1997). The Michaelis–Menten kinetics for nicotine C-oxidation show higher K_m and lower V_{max} values using liver microsome preparations from Japanese donors (Nakajima et al., 1996a), compared with other ethnic populations (Berkman et al., 1995; Messina et al., 1997).

It is generally accepted that CYP2A6[1] is the major CYP enzyme involved in the C-oxidation of physiological concentrations of nicotine (Messina et al., 1997). The lowest K_m values for nicotine C-oxidation are found for protein products encoded by human CYP2A6 cDNA, followed by CYP2B6 cDNA and CYP2D6 cDNA expressed in human B-lymphoblastoid cell lines (Nakajima et al., 1996a), cultured human hepatoma (HepG2) cells (Flammang et al., 1992), and in microsomes of *Trichoplusia ni* cells (Yamazaki et al., 1999). Human CYP2A6 cDNA expressed in human B-lymphoblastoid cells encodes a protein which catalyzes C-hydroxylation of cotinine to *trans*-3′-hydroxycotinine, albeit with a relatively high K_m (234.5 ± 26.8 μM) (Nakajima et al., 1996b). The Michaelis–Menten kinetics for in vitro metabolism of cotinine to *trans*-3′-hydroxycotinine by human liver microsomes varies about 20-fold (Nakajima et al., 1996b). Expression of human CYP2A6 cDNA in insect Sf9 cells also catalyzes the formation of 5′-hydroxycotinine, norcotinine, and an unidentified metabolite (Murphy et al., 1999).

2.1.1. CYP2A6 expression and polymorphism

CYP2A6 (coumarin 7-hydroxylase) is expressed primarily in the liver (Yun et al., 1991; Koskela et al., 1999) and nasal respiratory epithelia (Su et al., 1996; Koskela et al., 1999), and to a lesser extent in lung (Crawford et al., 1998; Macé et al., 1998) and other extrahepatic tissues (Degawa et al., 1994; Hellmold et al., 1998; Koskela et al., 1999; Lechevrel et al., 1999). Hepatic CYP2A6 activity is not affected by smoking (Pelkonen et al., 1986), but CYP2A6 mRNA expression in human bronchial epithelial cells appears to be significantly lower in smokers, compared with nonsmokers (Crawford et al., 1998).

In vitro studies using coumarin as a probe drug show marked interindividual variability in hepatic CYP2A6 activity in which some liver samples lack enzyme activity (Yun et al., 1991; Nakajima et al., 1996a,b; Shimada et al., 1996; Edwards et al., 1998). Liver samples from Japanese show significantly lower levels of CYP2A6 immunoreactivety and coumarin 7-hydroxylation activity compared with liver samples from Caucasians (Shimada et al., 1996). Phenotyping studies using coumarin as a probe drug also show interindividual variability in which some subjects show relatively low or no detectable excretion of 7-hydroxycoumarin (Iscan et al., 1994; Satarug et al., 1996; Rautio et al., 1997; Oscarson et al., 1998). Increased CYP2A6 enzyme activity and in vivo coumarin 7-hydroxylation is observed in subjects with a history of use of barbiturates (Cashman et al., 1992; Berkman et al., 1995) and some antiepileptic drugs

[1] Human CYP2A6 was previously named P450 2A3 (Yamano et al., 1989; Miles et al., 1990).

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(carbamazepine, clonazepam, and phenytoin) (Sotaniemi et al., 1995). Increasing age (Sotaniemi et al., 1996), viral hepatitis A infection (Pasanen et al., 1997) and parasitic infection by the liver fluke *Opisthorchis viverrini* (Satarug et al., 1996) impair in vivo CYP2A6 activity. Some evidence suggests that dietary factors may potentially modify CYP2A6 activity; both grapefruit juice flavonoids (Merkel et al., 1994) and wheat germ (Rauma et al., 1996) partially inhibit coumarin 7-hydroxylation in healthy volunteers. Phenotyping studies using caffeine as a probe drug provide additional evidence that CYP2A6 activity is not influenced by smoking status (Nowell et al., 2002).

The *CYP2A* gene cluster is mapped to chromosome 19q13.2 and contains five genes arranged in tandem; three complete genes (*CYP2A6*, *CYP2A7* and *CYP2A13*), and two truncated *CYP2A7* pseudogenes (*CYP2A7PC* and *CYP2A7PT*) (Fernandez-Salguero and Gonzalez, 1995). A two-step nested polymerase chain reaction (PCR) method combined with restriction fragment length polymorphism (RFLP) analysis has been reported to identify the wild-type allele (*CYP2A6*1*[2]) and two variant alleles (*CYP2A6*2* and *CYP2A6*3*) (Fernandez-Salguero et al., 1995). The *CYP2A6*2* allele contains a 479 T→A point mutation in codon 3 which leads to a L160H amino acid exchange. The *CYP2A6*3* allele contains multiple gene conversions in exons 3, 6, and 8 with the *CYP2A7* gene. The *CYP2A6*2* allele encodes for a catalytically inactive enzyme (Yamano et al., 1990) and homozygote *CYP2A6*2/*2* individuals lack coumarin 7-hydroxylation capacity in vivo (Hadidi et al., 1997; Oscarson et al., 1998). It has been suggested that the L160H substitution results in an enzyme with altered regiospecificity that catalyzes coumarin 3-hydroxylation instead of 7-hydroxylation (Hadidi et al., 1997). However, a more likely explanation for the absence of in vivo coumarin 7-hydroxylation is shunting of coumarin to the 3-hydroxylation pathway catalysed by other CYP enzymes (CYP1A1, CYP1A2, CYP2E1 and CYP3A4) in the absence of CYP2A6-dependent 7-hydroxylation (Zhou et al., 1999). The *CYP2A6*3* allele has been proposed to be inactive (Fernandez-Salguero et al., 1995), although this has never been demonstrated.

It was originally reported that significant ethnic differences occur in the frequencies of *CYP2A6*2* (0–20%) and *CYP2A6*3* (0–28%) alleles (Fernandez-Salguero et al., 1995; Gullstén et al., 1997; Nowak et al., 1998). More recently, several alternative assays have been developed to identify the *CYP2A6*2* variant by allele-specific PCR (Oscarson et al., 1998) and the *CYP2A6*2* and *CYP2A6*3* variants by one-step PCR–RFLP methods (Chen et al., 1999; Kitagawa et al., 1999; Sabol and Hamer, 1999; Tan et al., 2000; Zabetian et al., 2000; Paschke et al., 2001).

Using allele-specific PCR gives a low *CYP2A6*2* allele frequency (1.1–3.0%) in Caucasian populations and an accurate prediction of the CYP2A6 phenotype (Oscarson et al., 1998). Other studies using PCR–RFLP analysis confirm a *CYP2A6*2* allele frequency of 0.3–4.7% in African–American (Paschke et al., 2001) and Caucasian populations (Chen et al., 1999; Oscarson et al., 1999a,b; Sabol and Hamer, 1999; Zabetian et al., 2000; Loriot et al., 2001; Paschke et al., 2001; Rao et al., 2000; Schulz et al., 2001a,b; Xu et al., 2002). However, contrary to Chen et al. (1999), who reported a *CYP2A6*2* allele frequency of 0.7% in Chinese, nearly all recent studies report absence of the *CYP2A6*2* allele in Oriental populations (Kitagawa et al., 1999, 2001; Miyamoto et al., 1999; Oscarson et al., 1999a,b; Zabetian et al., 2000; Nakajima et al., 2001; Xu et al., 2002; Yang et al., 2001; Zhang et al., 2001).

The variant *CYP2A6*3* allele is now considered to be a genotyping artefact (Oscarson et al., 1999a), most probably caused by a gene conversion with the closely related *CYP2A7* gene in the 3′-flanking region of the *CYP2A6* gene that abolishes a binding site for one of the primers used in the PCR genotyping method of Fernandez-Salguero et al. (1995). Using improved PCR–RFLP genotyping methods, the *CYP2A6*3* allele is not found in African–American (Paschke et al., 2001), Caucasian (Oscarson et al., 1999a; Sabol

[2] Nomenclature of *CYP2A6* alleles according to recommendations by the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (see http://www.imm.ki.se/CYPalleles/).

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and Hamer, 1999; Gu et al., 2000; Paschke et al., 2001) or Oriental populations (Kitagawa et al., 1999, 2001; Miyamoto et al., 1999; Oscarson et al., 1999a; Kwon et al., 2001; Nakajima et al., 2001; Yang et al., 2001; Zhang et al., 2001). One study reports the presence of the *CYP2A6*3* allele in Chinese, albeit with a low allele frequency of 0.7% (Chen et al., 1999).

Recently developed genotyping methods have also established that the wild-type *CYP2A6*1* allele exists as two haplotypes, *CYP2A6*1A* and *CYP2A6*1B* (containing 58 bp of *CYP2A7* in the 3′-untranslated region), with similar catalytic activity (Oscarson et al., 1999a; Ariyoshi et al., 2000). Some *CYP2A6*1B* variants may have been misidentified as the *CYP2A6*3* allele using an earlier genotyping method (Fernandez-Salguero et al., 1995). The frequency of *CYP2A6*1A* and *CYP2A6*1B* alleles are almost identical (ca. 40%) in Oriental populations (Oscarson et al., 1999a; Ariyoshi et al., 2000; Kwon et al., 2001; Nakajima et al., 2001), compared with a frequency distribution of 66% *CYP2A6*1A* and 30% *CYP 2A6*1B* in Caucasians (Oscarson et al., 1999a; Ariyoshi et al., 2000). The functional significance of the *CYP2A6*1B* allele on CYP2A6 activity is unclear.

Several nonfunctional truncated *CYP2A6* alleles have been identified that lack DNA sequences and code for an inactive enzyme (Table 2). These deletion alleles are designated as *CYP2A6*4A* (Oscarson et al., 1999b; Nunoya et al., 1999a,b), *CYP2A6*4B* (Nunoya et al., 1998), *CYP2A6*4C* (Nunoya et al., 1999b), and *CYP2A6*4D* (Oscarson et al., 1999a). The *CYP2A6*4A* allele appears to be identical in sequence to *CYP2A6*4C* (Ariyoshi et al., 2000), while the *CYP2A6*4D* allele has only been identified in a single Caucasian subject (Oscarson et al., 1999a). The two major deletion alleles (*CYP2A6*4A* and *CYP2A6*4B*) typically occur with a frequency of 0.5–4% in Caucasians (Oscarson et al., 1999a,b; Ariyoshi et al., 2000; Rao et al., 2000; Loriot et al., 2001; Xu et al., 2002) and about 7–22% in Orientals (Oscarson et al., 1999a,b; Ariyoshi et al., 2000; Kwon et al., 2001; Nakajima et al., 2001; Tan et al., 2001; Xu et al., 2002), suggesting that the frequency of the *CYP2A6* 'poor metaboliser' (PM) phenotype is less than 1% in Caucasian and about 5% in Oriental populations (Yokoi and Kamataki, 1998; Oscarson et al., 1999b). The origins of these truncated alleles is thought to be due to unequal crossover events between the 3′-flanking regions of *CYP2A6* and *CYP2A7* genes (Nunoya et al., 1999b; Oscarson et al., 1999b). Thus, a *CYP2A6*4* deletion allele is formed together with an allele (*CYP2A6*1X2*) with two *CYP2A6* genes in tandem as the reciprocal outcome (Rao et al., 2000). In Caucasians the *CYP2A6*1X2* allele occurs with a frequency of 1.2–1.7% (Rao et al., 2000; Xu et al., 2002), slightly higher than in Orientals (Xu et al., 2002).

The *CYP2A6*5* allele contains a 1436 G→T point mutation in exon 9 resulting in a G479V amino acid exchange, as well as a gene conversion in the 3′-untranslated region (Oscarson et al., 1999a). The *CYP2A6*5* allele encodes an unstable enzyme which rapidly degrades to abrogate enzyme activity. This allele is very rare in Caucasians (Oscarson et al., 1999a) and occurs at a low frequency (0.5–1.0%) in Oriental populations (Oscarson et al., 1999a; Kwon et al., 2001; Nakajima et al., 2001). The *CYP2A6*6* allele, which contains a 383 G→A point mutation in exon 3 resulting in a R128Q amino acid exchange, occurs with a low frequency (0.4%) in Japanese, and codes for an enzyme with significantly reduced coumarin 7-hydroxylase activity (Kitagawa et al., 2001). In Japanese, two further point mutations, 1412 T→C and 1454 G→T, have been identified in exon 9 which result in amino acid exchanges at I471T (*CYP2A6*7*) and R485L (*CYP2A6*8*), respectively, (Ariyoshi et al., 2001; Xu et al., 2002). The *CYP2A6*7* allele appears to occur in association with the *CYP2A6*1B* allele with a calculated frequency of 15.7% in Japanese, while the *CYP2A6*8* allele frequency is rather low (2.5%). Slightly lower allele frequencies are found in Chinese (2.2 and 3.5% for *CYP2A6*7* and *CYP2A6*8*, respectively), while both alleles are absent in Caucasians (Xu et al., 2002). The *CYP2A6*7* allele encodes for an enzyme with decreased coumarin 7-hydroxylase activity, while no change in catalytic activity is associated with the *CYP2A6*8* allele (Xu et al., 2002). The *CYP2A6*9* allele contains a −48 T→G point

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Table 2
Current *CYP2A6* allele nomenclature

Allele	Nucleotide changes	Effect	Enzyme activity		Reference
			In vivo	In vitro	
CYP2A6*1A	None		Normal	Normal	Yamano et al., 1990
CYP2A6*1B	Gene conversion in the 3′-flanking region				Oscarson et al., 1999a
CYP2A6*1X2		*CYP2A6* gene duplication			Rao et al., 2000
CYP2A6*2	488 T→A	L160H	None	None	Yamano et al., 1990; Hadidi et al., 1997; Oscarson et al., 1998
CYP2A6*3[a]	CYP2A6/CYP2A7 hybrid				Fernandez-Salguero et al., 1995
CYP2A6*4A	*CYP2A6* deleted	*CYP2A6* deleted	None		Oscarson et al., 1999b; Nunoya et al., 1999a,b
CYP2A6*4B	*CYP2A6* deleted	*CYP2A6* deleted	None		Nunoya et al., 1998
CYP2A6*4C[b]					Nunoya et al., 1999b
CYP2A6*4D	*CYP2A6* deleted	*CYP2A6* deleted	None		Oscarson et al., 1999a
CYP2A6*5	1436 G→T	G479V	None	None	Oscarson et al., 1999a
CYP2A6*6	383 G→A	R128Q		Decreased	Kitagawa et al., 2001
CYP2A6*7	1412 T→C; gene conversion in the 3′-flanking region	I471T	Decreased	Decreased	Ariyoshi et al., 2001; Xu et al., 2002
CYP2A6*8	1454 G→T; gene conversion in the 3′-flanking region	R485L	Normal		Ariyoshi et al., 2001; Xu et al., 2002
CYP2A6*9	−48 T→G	TATA box		Decreased	Pitarque et al., 2001
CYP2A6*10	1412 T→C; 1454 G→T; gene conversion in the 3′-flanking region	I471T; R485L	Decreased		Xu et al., 2002
CYP2A6*11	670 T?C	S224P	Decreased	Decreased	Diago et al., 2002

According to recommendations by Antonarakis and the Nomenclature Working Group (1998) and the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (see http://www.imm.ki.se/CYPalleles/).

[a] Later identified as a PCR artefact (Oscarson et al., 1998).

[b] Identical to *CYP2A6*4A* (Ariyoshi et al., 2000).

mutation in the TATA box of the 5′-flanking region of *CYP2A6* which results in a 58% decrease in expression as tested using a reporter construct (Pitarque et al., 2001). The *CYP2A6*9* allele occurs with an allele frequency of 5–7% in Caucasians and approximately 16% in Chinese, and is the most common *CYP2A6* variant in Caucasians associated with a functional decrease in CYP2A6 enzyme activity (Pitarque et al., 2001). The *CYP2A6*10* allele contains both the 1412 T→C and 1454 G→T nucleotide changes found in *CYP2A6*7* and *CYP2A6*8*, respectively, (Xu et al., 2002). This allele occurs with a low frequency in Orientals (<1.6%) and is absent in Caucasians. The presence of both point mutations together in one allele results in decreased, possibly even total abrogation, of enzyme activity. The *CYP2A6*11* allele contains a 670 T→C point mutation in exon 5 resulting in a S224P amino acid exchange (Diago et al., 2002). The *CYP2A6*11* allele, which has only been detected in a single subject, codes for an enzyme with reduced coumarin 7-hydroxylase activity.

2.1.2. *CYP2A6* polymorphism and nicotine metabolism

Due to extensive in vitro evidence that CYP2A6 is the major enzyme involved in nicotine C-oxidation (Flammang et al., 1992; Nakajima et al., 1996a; Yamazaki et al., 1999), several studies have investigated whether polymorphism of CYP2A6 is functionally associated with differences in nicotine metabolism. Kinetic analysis shows higher K_m values and very low V_{max} to K_m ratios for nicotine C-oxidation by liver microsomes from heterozygote *CYP2A6*11*2* indivi-

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duals, compared with microsomes from individuals homozygous for wild-type *CYP2A6*1* alleles (Inoue et al., 2000). Preliminary studies involving heterologous expression of *CYP2A6*7* cDNA in *Escherichia coli* suggest that this allele is associated with decreased in vitro coumarin 7-hydroxylase activity and total abrogation of nicotine C-oxidase activity (Ariyoshi et al., 2001). Thus, individuals with a *CYP2A6*7* allele may also be PMs of nicotine.

Deficient nicotine C-oxidation was originally reported in a single subject before the possible influence of CYP2A6 polymorphism on nicotine metabolism was suggested (Benowitz et al., 1995). Subsequent genotyping of the index case, and another subject with deficient metabolism of nicotine to cotinine, revealed that the index case had a *CYP2A6*2/*2* genotype while the other subject with deficient nicotine metabolism had neither the *CYP2A6*2* allele nor *CYP2A6*4A* gene deletion (Benowitz et al., 2001).

In experimental studies with abstinent smokers, oral administration of a nicotine-containing gum and determination of the plasma cotinine–nicotine ratio 2 h later has been used as an index of nicotine metabolism (Nakajima et al., 2000). Using this method, absence of nicotine C-oxidation and plasma cotinine was found in homozygote *CYP 2A6*4B/*4B* Japanese (Nakajima et al., 2001) and Korean subjects (Kwon et al., 2001). In both abstinent Japanese and Korean smokers, subjects with a *CYP2A6*4B* allele (either *CYP2A6*1A/ *4B* or *CYP2A6*1B/*4B*) had lower mean cotinine–nicotine ratios than subjects without a *CYP2A6*4B* allele (*CYP2A6*1A/*1A*, *CYP 2A6*1A/*1B*, and *CYP2A6*1B/*1B* genotypes). Subjects with a *CYP2A6*1B* allele had a high capacity to metabolise nicotine to cotinine, compared with subjects lacking a *CYP2A6*1B* allele. In Japanese, but not Korean subjects, the differences in mean cotinine–nicotine ratios for each genotype were significant. However, very broad and overlapping cotinine–nicotine ratios were found for each genotype in both Japanese (range: 0–14.71) and Koreans (range: 0–143.9). In general, Korean smokers tended to have higher metabolic ratios than Japanese smokers regardless of genotype. Since, both the above studies used the same analytical method to determine plasma cotinine, lack of sensitivity of the applied method to determine trace levels of cotinine may partially explain why homozygote *CYP2A6*4B/*4B* subjects appear to totally lack nicotine C-oxidase activity. In another experimental study in which abstinent Japanese smokers smoked six cigarettes with an identical tar and nicotine delivery, significantly reduced urinary cotinine excretion was observed in six smokers with homozygous deletion of *CYP2A6*4B*, compared with five smokers homozygous for the wild-type *CYP2A6*1* allele (0.22 ± 0.08 vs. 1.50 ± 0.50 mg cotinine/24 h; $P < 0.001$) (Kitagawa et al., 1999). Therefore, homozygous deletion of *CYP2A6*4B* only partially abrogates nicotine C-oxidation in vivo consistent with the results of in vitro studies showing that other CYP enzymes also catalyse nicotine C-oxidation (Flammang et al., 1992; Nakajima et al., 1996a; Yamazaki et al., 1999).

As an alternative approach to using nicotine-containing gum and determination of plasma cotinine–nicotine ratios (Nakajima et al., 2000), the in vivo kinetics of nicotine metabolism have been determined by oral administration of 4 mg nicotine free base, formulated as the bitartrate salt, and measurement of the 6 h nicotine area under the blood plasma concentration–time curve (AUC) (Xu et al., 2002). Volunteer Oriental subjects were selected to include only individuals homozygous for the wild-type *CYP2A6*1* allele ($n = 6$), or combinations of variant alleles (*CYP2A6*1/*7* [$n = 2$], *CYP2A6*4A/*4A* [$n = 3$], *CYP2A6*4A/*10* [$n = 1$], *CYP2A6*7/*7* [$n = 1$], *CYP2A6*1/*8* [$n = 1$], and *CYP2A6*1/*10* [$n = 1$]). No significant difference in 6 h plasma nicotine AUC was evident between subjects homozygous for the wild-type *CYP2A6*1* allele and a subject with a *CYP2A6*1/*8* genotype; however, subjects with other genotypes maintained higher plasma nicotine concentrations. The highest plasma nicotine levels were observed in subjects with either the *CYP2A6*4A/*4A* or *CYP2A6*4/*10* genotypes. Reduced nicotine metabolism was associated with the presence of the *CYP2A6*7* allele, consistent with in vitro results (Ariyoshi et al., 2001), but not with the *CYP2A6*8* allele. The presence of the *CYP2A6*10* allele was associated with a signifi-

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cant reduction of in vivo nicotine C-oxidation to cotinine.

In voluntary smokers, blood plasma cotinine levels appear to reflect differences in *CYP2A6* genotype (Rao et al., 2000). In a study of 296 Caucasian smokers, mean blood plasma cotinine levels were significantly higher ($P < 0.05$) in smokers with *CYP2A6* gene duplication ($n = 5$, 378 ± 76 ng cotinine/ml), than in smokers either homozygous for wild-type *CYP2A6*1* alleles ($n = 277$; 265 ± 9 ng cotinine/ml) or smokers with either a *CYP2A6*2* or *CYP2A6*4A* allele ($n = 14$, 217 ± 76 ng cotinine/ml). However, self-reported smoking behaviour was almost identical in subjects with *CYP2A6* gene duplication (13.3 ± 3.3 cpd) and either a *CYP2A6*2* or *CYP2A6*4A* allele (13.5 ± 2.3 cpd), but significantly lower ($P < 0.05$) than in subjects homozygous for wild-type *CYP2A6*1* alleles (19.5 ± 0.7 cpd). Although urinary nicotine and cotinine were determined (Tyndale and Sellers, 2001), the data have never been reported.

Contrary to studies involving the determination of plasma cotinine levels after experimental administration of nicotine (Nakajima et al., 2000, 2001; Kwon et al., 2001) or smoking (Rao et al., 2000), population studies provide only limited evidence that *CYP2A6* polymorphism is associated with a shift in urinary nicotine metabolite profiles. In 157 European smokers with a self-reported consumption of 20 cpd, no evidence was found to support impaired metabolism of nicotine and decreased urinary excretion of cotinine in subjects with one *CYP2A6*2* allele, compared with smokers homozygous for wild-type *CYP2A6*1* alleles (Schulz et al., 2001a). In a Japanese study of 190 smokers, subjects with a *CYP2A6*4B/*4B* genotype had significantly lower excretion of urinary cotinine at each level of smoking (1–10, 11–20, and 21 < cpd), compared with smokers with a *CYP2A6*1* allele (Yang et al., 2001). Urinary cotinine excretion was significantly correlated ($r = 0.317$; $P < 0.001$) with the number of cigarettes smoked per day, and appeared to be attenuated in subjects with at least one *CYP2A6*1* allele and a *Rsa*I 5′-flanking polymorphism at nucleotide 1019 in *CYP2E1*, compared with smokers with at least one *CYP2A6*1* allele and homozygous for wild-type *CYP2E1*1* alleles.

2.1.3. CYP2A6 polymorphism and smoking behaviour

Based on the simple assumption that tobacco consumption is to some degree a reflection of nicotine dependence, the presence of variant *CYP2A6* alleles encoding for reduced enzyme activity and maintenance of high blood nicotine concentrations has been investigated in association with reduced smoking behaviour and tobacco consumption (Table 3). An association between *CYP2A6* genotype and self-reported smoking behaviour was first claimed in a study of alcohol and tobacco-dependent smokers (Pianezza et al., 1998). Dependence was defined using the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; American Psychiatric Association, 1994), and *CYP2A6* genotype determined using the two-step PCR method developed by Fernandez-Salguero et al. (1995). Carriers of at least one *CYP2A6*2* or *CYP2A6*3* allele were significantly underrepresented among alcohol and tobacco-dependent smokers ($n = 244$), compared with 184 never-tobacco-dependent control subjects who had tried smoking but had never become dependent (12.3 vs. 19.6%; $P < 0.04$). Among tobacco-dependent smokers ($n = 161$), heterozygote *CYP2A6*1/** subjects (where * was either *CYP2A6*2* or *CYP2A6*3*) smoked significantly fewer self-reported cigarettes per week (cpw), compared with subjects homozygous for wild-type *CYP2A6*1* alleles (mean 129 vs. 159 cpw; $P < 0.02$). Another study using the same genotyping method found no association between *CYP2A6*1/*1*, *CYP2A6*1/** and *CYP2A6*/** genotypes (where * was either *CYP2A6*2* or *CYP2A6*3*) and self-reported cigarette consumption (London et al., 1999). As described earlier, the two-step PCR genotyping method developed by Fernandez-Salguero et al. (1995) is prone to misidentification of some *CYP2A7* alles as *CYP2A6*2*, and some *CYP 2A6*1B* variants as *CYP2A6*3* alleles resulting in misclassification of the *CYP2A6* genotype. Indeed, repeating the *CYP2A6*2* allele genotyping of DNA samples from the original study by Pianezza et al. (1998) with allele-specific (Oscarson et al., 1998) and one-step PCR assay (Chen et al., 1999) resulted in a change in classification of nine

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Table 3
Population studies on *CYP2A6* polymorphism and self-reported smoking behaviour

Variant *CYP2A6* alleles	Effect on self-reported smoking behaviour	Reference
*CYP2A6*2* and *CYP2A6*3*	Significantly fewer subjects with at least one *CYP2A6*2* or *CYP2A6*3* allele among alcohol and tobacco-dependent smokers ($n=244$) compared with 184 never-tobacco-dependent controls (12.3 vs. 19.6%; $P<0.04$). Among tobacco-dependent smokers ($n=161$), heterozygote *CYP2A6*1/** subjects (where * is either *CYP2A6*2* or *CYP2A6*3*) smoked significantly fewer self-reported cigarettes per week (cpw), compared with homozygous *CYP2A6*1/*1* subjects (mean 129 vs. 159 cpw; $P<0.02$). Tobacco dependence was defined using the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; American Psychiatric Association, 1994) and *CYP2A6* genotype determined using the two-step PCR method developed by Fernandez-Salguero et al. (1995)	Pianezza et al., 1998
*CYP2A6*2* and *CYP2A6*3*	**None.** No significant difference in genotype frequencies (*CYP2A6*1/*1*, *CYP2A6*1/** and *CYP2A6*/**, where * is either *CYP2A6*2* or *CYP2A6*3*) between 299 male and female ever smokers and 161 never smokers. No significant difference between the mean number of self-reported cigarettes per day (cpd) and *CYP2A6* genotype. Genotyping performed according to the method of Fernandez-Salguero et al. (1995)	London et al., 1999
*CYP2A6*2* and *CYP2A6*3*	No significant difference in genotype frequencies (*CYP2A6*1/*1*, *CYP2A6*1/*2* and *CYP2A6*2/*2*) between current smokers ($n=142$), former smokers ($n=501$) and never smokers ($n=389$). No association between genotype and self-reported duration of smoking (years) or peak amount smoked calculated as cigarette equivalents per day. The *CYP2A6*2* allele was significantly associated with ever having smoked ($P<0.05$), initiation of regular smoking at an earlier age (17–18 vs. 20–21 years; $P<0.01$), and a lower likelihood of smoking cessation ($P<0.01$). The *CYP2A6*3* allele was not detected in 1032 subjects using a long PCR modification of the method of Fernandez-Salguero et al. (1995)	Gu et al., 2000
*CYP2A6*2* and *CYP2A6*3*	**None.** No significant difference in genotype frequencies (*CYP2A6*1/*1*, *CYP2A6*2/** and *CYP2A6*3/**, where * is either *CYP2A6*1*, *CYP2A6*2* or *CYP2A6*3*) between 126 male and female current smokers, 43 former smokers, and 189 nonsmokers of mixed ethnicity. No significant difference in mean self-reported cpw between heterozygous *CYP2A6*1/** and homozygous *CYP2A6*1/*1* smokers was found using either the genotyping method of Fernandez-Salguero et al. (1995) (149 ± 25 vs. 116 ± 7 cpw) or a newly developed one-step PCR method (100 ± 19 vs. 123 ± 8 cpw). The *CYP2A6*3* allele, present at an allele frequency of 7.3% using the method of Fernandez-Salguero, was not detected in 385 subjects using the new PCR method	Sabol and Hamer, 1999
*CYP2A6*2* and *CYP2A6*3*	**None.** No significant difference in genotype frequencies (*CYP2A6*1/*1* and *CYP2A6*1/*2*) between 357 self-reported European smokers and 556 nonsmokers. No significant difference found between urinary cotinine excretion and *CYP2A6* genotype. The *CYP2A6*3* allele was not detected in 913 subjects using the method of Chen et al. (1999)	Schulz et al., 2001a

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Table 3 (*Continued*)

Variant *CYP2A6* alleles	Effect on self-reported smoking behaviour	Reference
*CYP2A6*3*	None. No significant difference in genotype frequencies (*CYP2A6*1/*1*, *CYP2A6*1/*3* and *CYP2A6*3/*3*) between 226 Chinese smokers and 124 nonsmokers. No significant difference in self-reported amount smoked and *CYP2A6* genotype. The method used for genotyping was not stated, but presumed to be that of Fernandez-Salguero et al. (1995)	Tan et al., 2000
*CYP2A6*4A*	None. No significant difference in genotype frequencies (*CYP2A6*1/*1*, *CYP2A6*1/*4A* and *CYP2A6*4A/*4A*) between 174 Chinese smokers and 152 nonsmokers. No significant difference in daily self-reported tobacco consumption (<25 and ≥ 25 cpd) or duration of smoking (<20 and ≥ 20 years) and *CYP2A6* genotype. Genotyping performed according to the method of Oscarson et al. (1999b)	Tan et al., 2001
*CYP2A6*2*, *CYP2A6*4A*, and *CYP2A6*1X2*	Male and female Caucasian smokers with either a *CYP2A6*2* or a *CYP2A6*4A* allele (*CYP2A6*1/*2*, *CYP2A6*1/*4A* and *CYP2A6*2/*2*; $n = 14$) smoked significantly fewer self-reported cigarettes than 277 homozygous *CYP2A6*1/*1* smokers (13.5 ± 2.3 vs. 19.5 ± 0.7 cpd; $P < 0.03$). Subjects with *CYP2A6*1X2* gene duplication ($n - 5$) smoked less than homozygous *CYP2A6*1/*1* smokers (13.3 ± 3.3 vs. 19.5 ± 0.7 cpd; $P =$ NS). Genotyping for *CYP2A6*2* and *CYP2A6*4A* was performed according to the methods of Oscarson et al. (1998), 1999b). *CYP2A6* gene duplication determined as published	Rao et al., 2000 (preliminary results contained in an abstract by Tyndale et al., 2000)
*CYP2A6*2* and *CYP2A6*4A*	None. No significant difference in genotype frequencies (*CYP2A6*1/*1* and *CYP2A6*1/** , where * is either *CYP2A6*2* or *CYP2A6*4A*) between 285 smoking and 680 nonsmoking Caucasian men. No significant difference in self-reported cigarettes smoked between 268 homozygous *CYP2A6*1/*1* smokers and 17 heterozygous *CYP2A6*1/** individuals (18.4 ± 9.9 vs. 17.4 ± 6.2 cpd). Genotyping was performed according to the method of Oscarson et al. (1998)	Tiihonen et al., 2000
*CYP2A6*2* and *CYP2A6*4A*	None. No significant difference in genotype frequencies (*CYP2A6*1/*1* and *CYP2A6*1/** plus *CYP2A6*/**, where * is either *CYP2A6*2* or *CYP2A6*4A*) and smoking status in 219 Caucasian men. No significant difference in genotype frequencies and self-reported cigarette consumption (cpd), number of pack years, and duration of smoking (years) in 127 former and 56 current smokers. Genotyping of the *CYP2A6*2* allele was performed according to the method of Oscarson et al. (1998) and the *CYP2A6*4A* allele according to Miyamoto et al. (1999)	Loriot et al., 2001
*CYP2A6*2*, *CYP2A6*3*, and *CYP2A6*4B*	None. No significant difference in genotype frequencies (*CYP2A6*1/** and *CYP2A6*4B/*4B*, where * is either *CYP2A6*1* or *CYP2A6*4B*) and self-reported daily cigarette consumption (21.5 ± 8.9 vs. 16.2 ± 9.1 cpd) in 190 male Japanese smokers. Genotyping was performed according to the method of Kitagawa et al. (1999). *CYP2A6*2* and *CYP2A6*3* alleles not detected	Yang et al., 2001
*CYP2A6*2*, *CYP2A6*3*, and *CYP2A6*4B*	None. No significant difference in genotype frequencies (*CYP2A6*1/** and *CYP2A6*4B/*4B*, where * is either *CYP2A6*1* or *CYP2A6*4B*) between 96 Japanese male cigarette smokers and 141 nonsmokers. No significant difference in self-reported daily tobacco consumption (17.3 ± 1.0 vs. 18.4 ± 3.3 cpd) or duration of smoking (7.8 ± 0.7 vs. 6.4 ± 0.3 years) with *CYP2A6* genotype. Genotyping was performed according to the method of Kitagawa et al. (1999). *CYP2A6*2* and *CYP2A6*3* alleles not detected in 237 subjects	Zhang et al., 2001

Abbreviations used: cpw, cigarettes per week; cpd, cigarettes per day.

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subjects originally genotyped as *CYP2A6*2/*2* as *CYP2A6*1/*2*, and revision of the *CYP2A6*2* allele frequencies in both tobacco-dependent and never-tobacco-dependent controls (Rao et al., 2000). Consequently, any associations reported between *CYP2A6* genotype and smoking behaviour using the genotyping method of Fernandez-Salguero et al. (1995) must be viewed with caution.

Using a long PCR modification of the method of Fernandez-Salguero et al. (1995), no evidence for the *CYP2A6*3* allele was found in a representative population of 1032 European subjects (Gu et al., 2000). No significant difference in *CYP2A6*1/*1*, *CYP2A6*1/*2* and *CYP2A6*2/*2* genotype frequencies was observed in 142 current smokers, 501 former smokers, and 389 never smokers. However, the presence of the *CYP2A6*2* allele was significantly associated with ever having smoked ($P < 0.05$), initiation of regular smoking at an earlier age (17–18 vs. 20–21 years; $P < 0.01$), and a lower likelihood of smoking cessation at any time ($P < 0.01$). No association was found between *CYP2A6* genotype and self-reported peak cigarette consumption (determined in cigarette equivalents to take into account smoking of cigars, hand-made cigarettes and tobacco) in ever smokers. Contrary to the anticipated association that peak cigarette consumption should be reduced in subjects with a deficient variant *CYP2A6*2* allele, homozygous carriers of *CYP2A6*2* alleles had slightly, but not significantly, increased tobacco consumption. Using one-step PCR–RFLP assays, no associations are found between the presence of either *CYP2A6*1* or *CYP2A6*2* alleles and self-reported smoking status or cigarette consumption in a mixed US (Sabol and Hamer, 1999), European (Schulz et al., 2001b), and a Japanese population (Zhang et al., 2001).

One study (Tan et al., 2000) claimed no significant difference in the *CYP2A6*3* allele frequency between Chinese smokers (18%, $n = 226$) and nonsmokers (22.6%, $n = 124$); however, the genotyping method used was not described. No significant association was evident between *CYP2A6* genotype and self-reported amount smoked. The reported *CYP2A6*3* allele frequencies suggest that genotyping was performed using the method of Fernandez-Salguero et al. (1995), particularly as other studies report either a very low *CYP2A6*3* allele frequency (Chen et al., 1999) or absence of this allele in Chinese (Oscarson et al., 1999a,b). Thus, the validity of this study must also be viewed with caution.

The same working group which originally reported that carriers of *CYP2A6*2* and/or *CYP2A6*3* alleles show reduced self-reported cigarette consumption (Pianezza et al., 1998), has also claimed a similar association with the presence of *CYP2A6*2* and *CYP2A6*4A* alleles in Caucasians (Rao et al., 2000; Tyndale et al., 2000). Compared with 277 Caucasian smokers homozygous for the wild-type *CYP2A6*1* allele, heterozygote smokers with either a *CYP2A6*2* or a *CYP2A6*4A* allele ($n = 14$) had lower plasma cotinine levels (217 ± 76 vs. 265 ± 9 ng/ml) and significantly lower self-reported cigarette consumption (13.5 ± 2.3 vs. 19.5 ± 0.7 cpd; $P < 0.03$) (Rao et al., 2000). Exhaled breath carbon monoxide, another potential biomarker of cigarette smoke uptake, was also significantly lower in smokers with either a *CYP2A6*2* or a *CYP 2A6*4A* allele (14 ± 2 vs. 20 ± 0.6 ppm CO; $P < 0.05$). However, similar studies by other working groups report no association between the presence of *CYP2A6*2* and *CYP2A6*4A* alleles and self-reported cigarette consumption in Caucasians (Tiihonen et al., 2000; Loriot et al., 2001). The presence of *CYP2A6*2* and *CYP2A6*4A* alleles was also not associated with self-reported smoking status (nonsmoker, former smoker and current smoker), smoking duration in years, or pack-years of smoking history (Loriot et al., 2001). In Japanese, the presence of *CYP2A6*2* and *CYP 2A6*4B* alleles was not associated with smoking status or self-reported cigarette consumption (Zhang et al., 2001). In a Japanese study in which 190 smokers were genotyped as either '*CYP2A6*1*-positive' or *CYP2A6*4B/*4B* homozygotes, no difference in self-reported smoking behaviour (assessed as cpd) was observed (Yang et al., 2001). In Chinese, no association was evident between the *CYP2A6*4A* allele and self-reported daily tobacco consumption or duration of smoking (Tan et al., 2001).

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2.2. CYP2B6 and nicotine metabolism

CYP2B6 (*S*-mephenytoin *N*-demethylase) is expressed in the human liver, and expression varies widely between individuals (Code et al., 1997; Gervot et al., 1999). Immunoblot analysis shows only weak expression of CYP2B6 in brain, kidney, intestine and lung (Gervot et al., 1999). Using more sensitive quantitative competitive reverse transcriptase PCR amplification, no significant difference in expression is evident in bronchial epithelial cells of smokers and nonsmokers (Willey et al., 1997).

CYP2B6 is mapped to chromosome 19q12-13.2 (Hoffman et al., 1995), and is highly polymorphic (Lang et al., 2001). However, the functional significance of CYP2B6 polymorphism remains to be established. It is thought that the low hepatic levels of constitutive CYP2B6 play only a relatively minor role in the metabolism of xenobiotics (Elkins and Wrighton, 1999) including that of nicotine (McCracken et al., 1992; Yamazaki et al., 1999).

2.3. CYP2D6, nicotine metabolism and smoking behaviour

CYP2D6 (debrisoquine 4-hydroxylase) is expressed primarily in the liver (Zanger et al., 2001), and to a lesser extent in kidney, intestine and brain (Kivisto et al., 1997; Siegle et al., 2001). *CYP2D6* gene expression and immunoreactivity for CYP2D6 protein has not been detected in human lung (Kivisto et al., 1997; Macé et al., 1998).

CYP2D6, the only active gene in the human *CYP2D* gene cluster, is mapped to chromosome 22q13.1 (Gough et al., 1993). The molecular basis of the debrisoquine polymorphism is well characterised and PCR-based genotyping and allele-specific sequencing has identified 53 variant alleles at the *CYP2D6* gene locus (Marez et al., 1997). Many of the major variant alleles are specific to particular ethnic populations (Yokoi and Kamataki, 1998). Variable expression and function of CYP2D6 leads to distinct 'ultra-rapid' (UR), 'extensive' (EM), 'intermediate' (IM), and 'poor metaboliser' (PM) phenotypes. The PM phenotype is found in 5–10% of Caucasian and less than 1% of Oriental populations (Bertilsson et al., 1992; Yokoi and Kamataki, 1998). *CYP2D6* gene amplification present in the UR phenotype occurs in 1–2% of Caucasian populations (Saarikoski et al., 2000).

Oral administration of nicotine and determination of the urinary nicotine–cotinine ratio shows no association with *CYP2D6* genotype (Cholerton et al., 1994) or phenotype using dextromethorphan as a probe drug for CYP2D6 activity (Benowitz et al., 1996). No significant difference was found between self-reported cigarette consumption (measured as cpd) or number of years smoked between CYP2D6 PM and EM phenotypes (Benowitz et al., 1996). In fact, the in vivo kinetics of both nicotine and cotinine metabolism in CYP2D6 EM and PM phenotypes was similar suggesting that CYP2D6 is not a major enzyme for metabolism of both nicotine and cotinine (Benowitz et al., 1996).

Using debrisoquine as a probe drug, the CYP2D6 PM phenotype appears to be underrepresented among Caucasian cigarette smokers, compared with nonsmokers (Turgeon et al., 1995). However, the small size of this study (58 smokers and 200 nonsmokers) limits its ability to accurately assess the CYP2D6 phenotype distribution in smokers. The debrisoquine phenotype is not associated with pack-years of smoking history (Oates et al., 1987). In a study of 261 subjects of mixed origin which used dextromethorphan as a probe drug, the *CYP2D6* phenotype was unrelated to nicotine metabolite deposition (determined as nicotine, cotinine and *trans*-3′-hydroxycotinine excretion in urine), nicotine dependence assessed using the Fagerstrom score, and smoking behaviour (Caporaso et al., 2001). However, subjects with a UR phenotype exhibited a significantly lower nicotine–cotinine + *trans*-3′-hydroxycotinine ratio after multiple adjustment for various demographic factors and nicotine yield of cigarette smoked. The debrisoquine phenotype is not associated with pack-years of smoking history (Oates et al., 1987). No statistically significant differences in *CYP2D6* genotypes are apparent in European cigarette smokers and non-tobacco users (Cholerton et al., 1996). Furthermore, no association is evident between *CYP2D6* genotypes predicted to

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result in a PM phenotype and decreased smoking behaviour assessed as the number of cigarettes smoked per day (Boustead et al., 1997; Vincent-Viry et al., 2000). Contrary to this, one study reports that the prevalence of the *CYP2D6* UR metaboliser phenotype is significantly overrepresented among heavy smokers (defined as >20 cpd for at least 20 years), compared with self-reported never-smokers (Saarikoski et al., 2000).

3. Metabolism by N′-oxidation

Nicotine has two nitrogen centres that are susceptible to chemical oxidation to isomeric nicotine N-oxides; however, only the alicyclic pyrrolidine nitrogen is subject to metabolic oxidation (Booth and Boyland, 1971). Hepatic NADPH-dependent flavin-containing monooxygenase form 3 (FMO3), the major functional FMO form present in adult liver (Lang et al., 1998), appears to be the principal enzyme involved in stereoselective *N*-1′-oxidation of nicotine to produce exclusively the *trans*-(*S*)-nicotine *N*-1′-oxide (1′*S*,2′*S*-nicotine *N*-1′-oxide) in man (Cashman et al., 1992). Nicotine *N*-1′-oxide formation is not catalysed by human CYPs expressed in HepG2 cells (Flammang et al., 1992). Under in vitro conditions, human liver microsomes show a 7-fold difference in nicotine *N*-1′-oxide formation (Cashman et al., 1992). Both cigarette smoking and administration of intravenous or transdermal (*S*)-nicotine result only in the formation and excretion of the *trans* diastereomer of (*S*)-nicotine *N*-1′-oxide in urine (Park et al., 1993). At present it is not known whether cotinine *N*-1-oxidation is also mediated by FMO3.

Multiple point mutations have been characterized in the human *FMO3* gene which occur with significant heterogeneity in allele frequency, haplotypes and genotypes among different ethnic populations (Cashman et al., 2001). Although rare, some mutations which either reduce or abrogate *N*-oxygenase activity have been causally associated with trimethylaminuria (fish-like odour syndrome) (Cashman et al., 1997; Dolphin et al., 1997; Akerman et al., 1999; Forrest et al., 2001). The FMO3 PM phenotype associated with server trimethylaminuria is very uncommon although the true incidence and prevalence are difficult to estimate. One experimental study involving two siblings with clinical evidence of trimethylaminuria found impaired urinary excretion of nicotine *N*-1′-oxide following oral administration of nicotine via a chewing gum preparation (Ayesh et al., 1988).

4. Metabolism by *N*- and *O*-glucuronidation

Glucuronides of nicotine, cotinine, and *trans*-3′-hydroxycotinine are major urinary metabolites of nicotine in man (Curvall et al., 1991). These glucuronides have been structurally characterized as (*S*)-(−)-nicotine-*N*-1-β-glucuronide (Seaton et al., 1993), (*S*)-(−)-cotinine-*N*-1-β-glucuronide (Caldwell et al., 1992), and *trans*-3′-hydroxycotinine-*O*-β-D-glucuronide (Schepers et al., 1992). The extent of both *N*- and *O*-glucuronidation show a high interindividual variability in both adults (Byrd et al., 1992, 1995a, 1998; Benowitz et al., 1994, 1999; Andersson et al., 1997; Hecht et al., 1999) and newborns (Dempsey et al., 2000). In both smokers and subjects using transdermal nicotine, the extent of *N*-glucuronidation of nicotine and cotinine is highly correlated, but neither is correlated with the extent of *O*-glucuronidation of *trans*-3′-hydroxycotinine (Benowitz et al., 1994). Furthermore, cigarette smoking induces *O*-glucuronidation of *trans*-3′-hydroxycotinine, but not *N*-glucuronidation of either nicotine or cotinine (Benowitz and Jacob, 2000). *N*-Glucuronidation of nicotine and cotinine are significantly lower in African–Americans, compared with Caucasians; but there appears to be no ethnic differences in the extent of *trans*-3′-hydroxycotinine *O*-glucuronidation (Benowitz et al., 1999). These observations suggest that similar uridine diphosphate-glucuronosyltransferase (UGT) enzyme(s) are involved in the *N*-glucuronidation of nicotine and cotinine, and that a different UGT enzyme is involved in the *O*-glucuronidation of *trans*-3′-hydroxycotinine.

Over 20 human UGT family 1 (UGT1A) and family 2 (UGT2B) isoforms have been identified which catalyse the conjugation of glucuronic acid to lipophilic xenobiotics (Mackenzie et al., 2000). Of the currently identified UGTs, polymorphisms

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in UGT1A1, UGT1A6, UGT1A7, UGT2B4, UGT2B7 and UGT2B15 have been described (Guillemette et al., 2000; Lampe et al., 2000; Mackenzie et al., 2000). Of these enzymes, only UGT1A3 and UGT1A4 catalyse quaternary ammonium-linked glucuronide formation (Green and Tephly, 1998). The UGT isoform(s) involved in phase II metabolism of nicotine have not been characterized. However, it has been suggested that the strong correlation observed between *O*-glucuronidation of *trans*-3′-hydroxycotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in smokers supports the involvement of a common enzyme (Hecht et al., 1999). Since *O*-glucuronidation of NNAL is catalysed by human liver UGT1A9 and UGT2B7 (Ren et al., 2000), these two UGT enzymes may also be involved in the *O*-glucuronidation of *trans*-3′-hydroxycotinine. Although a *UGT2B7* H268Y polymorphism commonly occurs in Caucasians and Orientals (Lampe et al., 2000), excretion of *trans*-3′-hydroxycotinine *O*-glucuronide appears to be unimodal (Benowitz et al., 1999).

5. Discussion

It is not unreasonable to hypothesise that genetic polymorphisms may influence an individual's smoking behaviour. Candidate polymorphic genes include those involved in dopamine biosynthesis and metabolism, dopamine receptors, nicotinic acetylcholinergic receptors and enzymes involved in nicotine metabolism (Rossing, 1998). Variation in genes that encode enzymes responsible for chemical metabolism generally result from point mutations or small deletions/insertions that may lead to alterations in function of the enzyme (Kalow and Grant, 1995). Most genetic variation can be accounted for by a few variant alleles which, may vary widely in frequency in different ethnic populations.

At physiological concentrations of nicotine the major enzyme involved in nicotine C-oxidation is CYP2A6 (Messina et al., 1997). Polymorphism of CYP2A6 is associated with differences in nicotine C-oxidation in vitro (Inoue et al., 2000; Ariyoshi et al., 2001), and plasma cotinine levels after experimental administration of nicotine (Kwon et al., 2001; Nakajima et al., 2001; Xu et al., 2002) and voluntary smoking (Rao et al., 2000). The presence of the *CYP2A6*4B* deletion allele in Japanese subjects is significantly associated with reduced urinary excretion of cotinine; both after controlled experimental smoking (Kitagawa et al., 1999) and voluntary smoking (Yang et al., 2001). After correction for the number of cigarettes smoked, homozygous Japanese carriers of *CYP2A6*4B* alleles also show significantly lower urinary excretion of cotinine compared with subjects with a *CYP2A6*1* allele (Yang et al., 2001). Kinetic studies with Japanese and Chinese volunteers confirm that the presence of the *CYP2A6*4A* deletion allele, as well as the *CYP2A6*7* and *CYP2A6*10* alleles, are associated with reduced in vivo nicotine metabolism to cotinine (Xu et al., 2002). The high frequency of *CYP2A6*4* deletion alleles and the *CYP2A6*7* allele in Orientals compared with other ethnic populations suggests that these alleles are likely to have a significant impact on nicotine metabolism in Oriental populations.

Inconsistent associations are reported for polymorphism of CYP2A6 and self-reported smoking behaviour (Table 3). The presence of either *CYP2A6*2* or *CYP2A6*3* variant alleles has been claimed to be associated with reduced self-reported smoking behaviour in one study (Pianezza et al., 1998). However, another working group using the same genotyping method could not confirm this observation (London et al., 1999). The alleged presence of the *CYP2A6*3* allele is now considered to be a genotyping artefact (Oscarson et al., 1999a). The *CYP2A6*2* allele is significantly associated with ever having smoked, initiation of regular smoking at an earlier age, and a lower likelihood of smoking cessation (Gu et al., 2000), but not with self-reported cigarette consumption in mixed US (Sabol and Hamer, 1999), European (Gu et al., 2000; Schulz et al., 2001a,b), and Japanese populations (Yang et al., 2001; Zhang et al., 2001). Similarly, the observed association between either *CYP2A6*2* or *CYP2A6*4A* variant alleles and reduced self-reported consumption of cigarettes in Caucasians (Rao et al., 2000) lacks confirmation in other studies (Loriot et al.,

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2001; Tiihonen et al., 2000). The presence of *CYP2A6*2* and *CYP2A6*4B* alleles is also not associated with reduced self-reported smoking behaviour in Japanese (Yang et al., 2001; Zhang et al., 2001). However, direct comparisons between most of these studies are difficult to make since different measures of smoking behaviour were assessed.

Current evidence suggests that multiple variant *CYP2A6* alleles exist with a low frequency in most populations, mainly as a consequence of unequal crossover events and/or gene conversions between *CYP2A6* and *CYP2A7* genes (Nunoya et al., 1999b; Oscarson et al., 1999b). It is evident that future studies will require better structural characterisation of putative new variant alleles and design of genotyping methods to avoid genotype misclassification as has previously occurred with misidentification of some *CYP2A7* alles as *CYP2A6*2*, and some *CYP2A6*1B* variants as *CYP2A6*3* alleles in early studies investigating CYP2A6 polymorphism and smoking behaviour (Pianezza et al., 1998; London et al., 1999). It is anticipated that the development of more specific high-throughput genotyping methods will result in the identification of a number of additional polymorphisms in the *CYP2A6* gene. Although it is likely that additional alleles will be discovered which functionally affect CYP2A6 activity, carefully designed phenotyping studies will be required to confirm whether these alleles have a significant effect on interindividual differences in nicotine metabolism. Phenotyping studies suggest that the major variant alleles coding for the PM phenotype have already been identified. If nicotine is the sole determinant of an individual's smoking behaviour (Russell and Feyerabend, 1978) and initial blood nicotine depletion is CYP2A6-dependent, the lack of concordance between plasma cotinine levels and *CYP2A6* genotype with self-reported smoking behaviour are hard to explain (Rao et al., 2000).

At high nicotine concentrations, far above physiological concentrations, both CYP2B6 and CYP2D6 also metabolise nicotine by C-oxidation. Although CYP2B6 is highly polymorphic (Lang et al., 2001), no investigation of CYP2B6 polymorphism and either nicotine metabolism or smoking behaviour has been reported. No conclusive evidence exists to show that CYP2D6 polymorphism is associated with either differences in nicotine metabolism (Cholerton et al., 1994; Benowitz et al., 1995, 1996; Caporaso et al., 2001) or self-reported smoking behaviour (Cholerton et al., 1996; Boustead et al., 1997; Vincent-Viry et al., 2000; Caporaso et al., 2001). One exception is the suggested overrepresentation of the CYP2D6 UR phenotype among heavy smokers, compared with never-smokers (Saarikoski et al., 2000). In general, the observed lack of association between *CYP2D6* genotype/phenotype and in vivo nicotine metabolism is consistent with nicotine not being a major substrate for CYP2D6 metabolism (Benowitz et al., 1996; Sellers, 1998). Consequently, polymorphism of CYP2D6 is probably of little importance as a determinant of smoking behaviour.

FMO3 appears to be the principal enzyme involved in stereoselective *N*-1′-oxidation of nicotine to *trans*-(*S*)-nicotine *N*-1′-oxide (Cashman et al., 1992). Only limited data support decreased formation of nicotine *N*-1′-oxide in two siblings with clinical evidence of trimethylaminuria, a metabolic deficit associated with FMO3 polymorphism (Ayesh et al., 1988). Both the minor role of nicotine *N*-oxidation in total nicotine metabolism, and the rare occurrence of the FMO3 PM phenotype, suggest that polymorphism of FMO3 is unlikely to contribute significantly to differences in an individual's smoking behaviour.

The UGT enzymes involved in the formation of nicotine-*N*-1-β-glucuronide, cotinine-*N*-1-β-glucuronide, and *trans*-3′-hydroxycotinine-*O*-β-D-glucuronide remain to be conclusively identified. Compared with nicotine C-oxidation, formation of nicotine-*N*-1-β-glucuronide is only a very minor pathway of nicotine metabolism accounting for only 4% of total nicotine metabolism (Fig. 1). It is unlikely that polymorphism of the enzyme(s) involved in nicotine *N*-glucuronidation play any significant role in total nicotine disposition and depletion of blood nicotine levels, particularly as the Michaelis–Menten kinetics for hepatic nicotine *N*-glucuronidation (Ghosheh et al., 2001) are unfavourable compared with those for C-oxidation (Messina et al., 1997), the predominant pathway of nicotine depletion. Similarly, phase II metabolism of cotinine and *trans*-3′-hydroxycoti-

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nine to cotinine-*N*-1-β-glucuronide and *trans*-3′-hydroxycotinine-*O*-β-D-glucuronide, respectively, will not affect initial CYP2A6-dependent nicotine depletion. Thus, it is very unlikely that polymorphic differences in the formation and excretion of nicotine-*N*-1-β-glucuronide, cotinine-*N*-1-β-glucuronide, and *trans*-3′-hydroxycotinine-*O*-β-D-glucuronide are determinants of an individual's smoking behaviour.

In summary, current evidence does not consistently and conclusively support the hypothesis that genetic polymorphisms in genes involved in the metabolism and depletion of blood nicotine concentrations are a determinant of an individual's smoking behaviour. However, it is recommended that future studies investigating metabolic gene polymorphisms implicated in the metabolism of nicotine and differences in smoking behaviour should use better validated genotyping methods combined with the determination of nicotine and its major metabolites including glucuronide conjugates in urine collected over 24 h, rather than the determination of nicotine and/or cotinine alone in human body fluids, as an estimate of total nicotine uptake and exposure.

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